Neuron, Vol. 17, 1031–1034, December, 1996, Copyright ©1996 by Cell Press

Axons Turn as Netrins Find Their Receptor

Minireview

Roger Keynes and Geoffrey M. W. Cook
Department of Anatomy
University of Cambridge
Cambridge CB2 3DY
United Kingdom

The idea of chemoattraction as a guiding mechanism for growing axons was originally suggested by Ramon y Cajal as a result of earlier work on leucocytes. Discussing the ventral navigation of commissural axons toward the midline floor plate in the developing spinal cord, he wrote: "...The oblique direction assumed by these axons would be explained if chemoattractants produced by the ventral half of the neuroepithelium were stronger than those produced by the rest of the epithelium...a sufficiently extended period of chemoattractant secretion by the floor plate could explain the relatively long period of time over which the ventral commissure is formed" (Ramon y Cajal, 1909).

Chemoattraction is well established at the molecular level in leucocyte biology (reviewed by Schall and Bacon, 1994), but good evidence was lacking in the vertebrate nervous system until collagen gels were used to assay for chemoattractants (Lumsden and Davies, 1983; Tessier-Lavigne et al., 1988), allowing the purification and cloning of the netrins (Serafini et al., 1994; Kennedy et al., 1994). The homology then revealed between the netrins and UNC-6, a laminin-related protein required for circumferential dorsal and ventral migrations of axons in the body wall of Caenorhabditis elegans (Hedgecock et al., 1990; Ishii et al., 1992), further suggested that netrins may provide highly conserved midline guidance cues for axons, operating in organisms ranging from nematode worms to higher vertebrates. This appealing view is now amply confirmed with an impressive group of studies using worms, flies, and rodents to analyze neural development in the face of netrin loss- and gain-of-function, and the story has simultaneously taken a significant step forward with the identification of the structure and function of netrin receptors.

Vertebrates

Serafini et al. (1996) describe the phenotype of *netrin-1* loss-of-function in the mouse, using a gene trap vector to select for mutant genes encoding secreted proteins (Skarnes et al., 1995). The resulting chimeric transmembrane protein retains some aminoterminal *netrin-1* sequences in the extracellular domain, and very low levels of wild-type transcript are detectable in homozygotes following transmission of the gene through the germ line, so the mutant allele is severely hypomorphic rather than a complete null. Homozygotes fail to move their forelimbs independently, and die within a few days of birth, apparently due to defective suckling. In the embryonic spinal cord, the ventral commissure is greatly reduced. Commissural axons project ventrally from their cell bodies in the dorsal spinal cord, but midway down the dorsoventral axis they deviate from their normal course toward the floor plate, some going medially toward the lumen of the neural tube, some passing ventrally along the lateral surface of the cord, and some dropping vertically to invade the motor column (Figure 1). Consistent with the loss of netrin function, mutant floor plates also fail to elicit commissural outgrowth from the rat dorsal spinal cord in collagen gels.

Previous studies have indicated that the role of netrins in providing midline guidance cues extends rostral to the spinal cord (Shirasaki et al., 1995), and this is strikingly underscored in the *netrin-1* knockout mouse, whose major forebrain commissures, the corpus callosum, hippocampal and anterior commissures, are completely absent. The additional absence of pontine nuclei raises the possibility that netrin-1 is a chemoattractant for migrating cells as well as axon growth cones.

The homology of the netrins to UNC-6 suggested that the vertebrate homologs of the putative UNC-6 receptor involved in ventral migrations, UNC-40 (see below), might be netrin receptors. Two such genes, *DCC* and *neogenin*, have been identified, and their status as netrin receptors has now been confirmed by Keino-Masu et al. (1996). The *DCC* (deleted in colon carcinoma) gene was originally characterized as a candidate tumour suppressor gene that is frequently lost in human colorectal carcinomas (Fearon et al., 1990), and encodes a transmembrane protein which, together with neogenin, forms a subgroup of the immunoglobulin superfamily. Although both genes were known to be expressed in the developing nervous system in a variety of vertebrates, their function here has been somewhat speculative (e.g., Cooper et al., 1995; Vielmetter et al., 1994). The expression patterns of DCC in diverse mature epithelia have also suggested a role in regulating cell proliferation and/or differentiation (Cho and Fearon, 1995).

In the developing rat spinal cord, Keino-Masu et al. (1996) now find that *DCC* transcripts are strongly expressed by the cell bodies of commissural neurons (and motor neurons), but not by undifferentiated neuroepithelial cells. DCC protein is expressed on commissural axons as they project toward the floor plate, and on their growth cones in vitro, and appears to be a high affinity receptor as judged by netrin-1 binding to *DCC*-transfected cells. Direct involvement of DCC in axon growth was further verified using a monoclonal antibody against the extracellular domain of DCC, which inhibited netrin-1- and floor plate-evoked outgrowth of commissural axons in collagen gel explants.

It will be interesting to see whether, as predicted, the phenotype of *DCC* null mutant mice is similar to the netrin knockout phenotype. DCC may also have functions beyond mediating netrin-1 signals. In C. elegans, its homolog UNC-40 is required for some UNC-6- independent migrations, and DCC is expressed on certain axons (e.g., posterior commissure and motor axons) that do not appear to be netrin-1-responsive even though they are floor plate–responsive (Shirasaki et al., 1996; Tucker et al., 1996). The fact that some commissural axons do reach the floor plate in the netrin knockout also suggests that additional attractive guidance cues, distinct from netrin-1, are expressed by the floor plate. Consistent with this, netrin-1-mutant floor plate explants remain capable of reorienting commissural axons in coexplanted dorsal spinal cord, and the DCC antibody

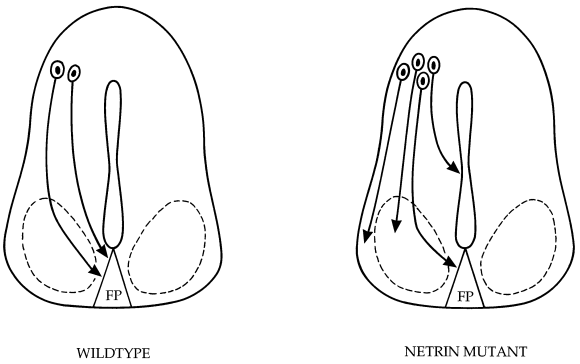

Figure 1. Commissural Axon Trajectories in the Spinal Cord

Schematic diagram of dorsoventral commissural axon trajectories in the spinal cord of wild-type (left) and netrin-mutant (right) mice. Dashed lines delineate regions of motor neuron cell bodies; FP, floor plate.

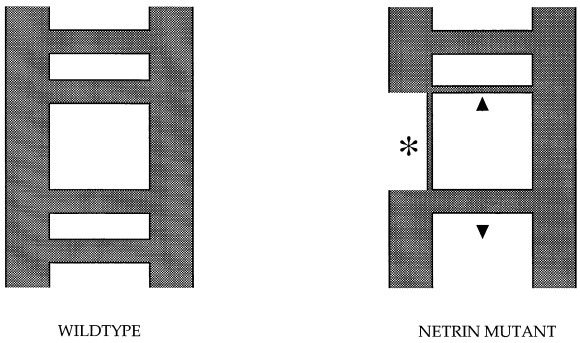

Figure 2. Two Segments of the Drosophila Nervous System in Wild-Type and *netrin* Knockout Flies

Schematic diagram of Drosophila CNS, represented at two consecutive segmental levels as a ladder-like arrangement of longitudinal (vertical) and commissural (horizontal) axons. In the wild-type (left), each segment contains two (anterior and posterior) commissures. Defects found in *netrin* mutants are shown on the right. Commissures are often thin (upper arrowhead) and sometimes absent (lower arrowhead). Occasional breaks are also seen in the longitudinal connectives (asterisk).

fails to block reorientation induced in vitro by normal floor plate or netrin-1-transfected cells.

The function of neogenin in neural development is less clear at present. The finding that it binds netrin-1, and is expressed on chick retinal ganglion cell axons, whereas netrin-1 is expressed in the developing optic stalk, suggests an involvement in retinal axon guidance. In the spinal cord, its expression in a pattern complementary to DCC has also led Keino-Masu et al. (1996) to suggest that it may stabilize the gradient of netrin-1 by acting as a passive binding protein.

Flies

The study of netrin function has now been extended from worms and vertebrates to flies, being joined by three studies providing excellent evidence that netrins function at the midline of Drosophila in a manner homologous to the body wall of C. elegans and the vertebrate spinal cord. Mitchell et al. (1996) and Harris et al. (1996) have identified two tandem netrin genes in Drosophila, both expressed (in slightly differing patterns) by cells of the developing CNS midline during the stages of commissure formation. Commissures are thin or absent in embryos carrying a deficiency that deletes both genes (Figure 2), a phenotype that can be rescued by expression of cDNA for either gene at the midline. Commissures are also thinned in embryos misexpressing either gene throughout the CNS using the GAL4–UAS system, conditions that are presumed to disrupt critical spatial distributions of netrin protein. This demonstrates elegantly that it is the correct pattern of netrin expression, and not simply the presence of expression itself, that is essential for netrins to function as instructive guidance cues for axons. Whether such concentration changes are step gradients at the cell surface, or smoother gradients in the extracellular matrix (as envisaged for vertebrates; Colamarino and Tessier-Lavigne, 1995), is at present unclear.

Strong evidence for the netrin hypothesis has also come from the study of Kolodziej et al. (1996), who have identified a *DCC* homolog in Drosophila, *frazzled*, in an enhancer trap screen, and studied its role in axon guidance. Like DCC and UNC-40, the *frazzled* product is an immunoglobulin superfamily member with four C2 domains and six fibronectin type III repeats, and is expressed by developing CNS and motor axons. The midline phenotype of the *frazzled* null mutant is identical to that of the netrin-deficient embryos, with thin or absent commissures (Figure 2), and can be rescued by pan-neural but not pan-muscular expression of *frazzled*, consistent with its presumptive role as a netrin receptor.

A further important role for netrins in targeting motor axons to their muscles has been revealed by these studies in Drosophila. Both *netrin* genes are expressed in differing subsets of segmental muscles, and in *netrin* as well as *frazzled* null mutants the intersegmental axons project inappropriately across segment boundaries into adjacent muscle territories. Following misexpression of either *netrin* gene in all muscles, axons also project and branch aberrantly, sometimes stalling short of their target muscles. These observations raise the interesting possibility of a homologous function for netrins in guiding motor axons to their segmental trunk and limb muscles in vertebrate embryos, although the insensitivity of spinal motor axons to netrin-1 in vitro (despite their expression of DCC, see above) argues against this. The fine detail of netrin/receptor distribution in developing worms, flies and vertebrates, and the finding that occasional breaks in the longitudinal CNS tracts are seen in both *frazzled* and *netrin* null mutants in flies (Figure 2), also suggest that the netrin/receptor system may contribute to axon pathfinding in anatomical regions beyond the midline and muscles.

Worms

A variety of cells and growth cones migrate along the inner surface of the epidermal body wall in C. elegans, and the products of three genes, *unc-5*, *unc-6*, and *unc-40*, are essential for the guidance of circumferential migrations in the dorsoventral axis. Genetic analysis has led to a model in which migrations are oriented either ventrally, toward a source of UNC-6 protein, or dorsally away from it, depending on the cell type and developmental stage. UNC-5 is a cell surface receptor that orients dorsal migrations, while UNC-40 expression is required for both ventral and some dorsal migrations, as

well as some UNC-6-independent migrations (reviewed by Culotti, 1994).

The expression of UNC-6 protein during early development has been mapped in detail recently by introducing an epitope tag into the cloned *unc-6* gene just after the signal peptide sequence (Wadsworth et al., 1996). Early expression is restricted to ventral cells (epidermoblasts, cephalic sheaths, and ventral midline neurons), and Wadsworth et al. suggest that a gradient of protein could be established on the body wall by ventral epidermoblasts secreting UNC-6 as they slide ventrally over the neuroectoderm. Later in development local, cell-specific patterns of UNC-6 expression also provide more restricted guidance cues for cells and axons.

Chan et al. (1996) have now cloned *unc-40* and find that it encodes a nematode homolog of *DCC*, *neogenin*, and *frazzled*. Using GFP fusion constructs, they find that UNC-40 is expressed on the surfaces of the same population of cells and neurons whose migrations are perturbed in *unc-40* mutants. UNC-40 also acts cell autonomously: its selective expression in mechanosensory neurons using the *mec-7* promoter substantially corrects their pathfinding defects in *unc-40* mutants, but not those of UNC-40 nonexpressing cells.

Since dorsal as well as ventral migrations are disrupted in *unc-40* mutants, UNC-40 expression is presumed to be necessary for both repulsive and attractive responses to UNC-6, and Chan et al. find that UNC-40 is indeed expressed by dorsally migrating cells. The simplest model is that UNC-40 and UNC-5 act as repulsion coreceptors for UNC-6 ligand. However, since dorsal migrations are less disrupted in *unc-40* null mutants compared with *unc-5* and *unc-6* nulls, Chan et al. suggest that this function of UNC-40 may be redundant to a second pathway, possibly involving a distinct DCC homolog. Some axons are also suggested by Wadsworth et al. to be consecutively attracted and repelled, or vice versa, by UNC-6, and it will be interesting to see how the timing of UNC-40 and UNC-5 expression correlates with such turning decisions.

Repulsion by Netrins in Worms, Flies, and Vertebrates

The earlier analysis of defective dorsal migrations in C. elegans mutants, and the chemorepulsion of rat trochlear motor axons by floor plate explants and netrin-transfected cells in collagen gels, indicated that netrins could have repulsive as well as attractive roles in cell migration and axon guidance. The new findings that the dorsal trajectory of trochlear axons is unperturbed in the *netrin* and *DCC* knockout mice, and that chemorepulsion of trochlear axons is maintained by netrin-deficient floor plate explants, leaves a repellent role for the vertebrate netrins as an open possibility. On the other hand, the data in flies are suggestive of netrin-based repulsion. Following pan-neural expression of either *netrin* gene, large bundles of axons project aberrantly, perhaps being repelled, toward the lateral edge of the CNS, and a subset of *frazzled*-expressing intersegmental axons also fail to enter their normal muscle territory when these muscles misexpress netrin (Mitchell et al., 1996). The fact that this axon population projects normally in netrin-deficient embryos suggests that, here at least, netrin repulsion might be a redundant mechanism.

Finally, and illustrating nicely the resolution of C. elegans mutant analysis in determining detailed structure/function relationships within guidance molecules, Wadsworth et al. (1996) have identified the second EGF-like repeat of UNC-6 as being critical in mediating dorsal migrations, perhaps by providing a binding site for the repulsion receptor UNC-5.

Conclusions

A recurrent theme of recent years has been the similarity between the molecular mechanisms that control pattern formation in vertebrates and invertebrates. While in some cases the same regulatory proteins and pathways carry out divergent functions in different organisms, the new data illuminate an outstanding example of a conserved ligand–receptor system operating with little apparent redundancy at the body midline. They also show how the individual experimental advantages of worms, flies and vertebrates can be put to good effect. Many interesting questions are raised, for example whether DCC-like proteins constitute a complete receptor system or an essential ligand-binding component, the relation between UNC-40 and UNC-5 in mediating repulsion, the existence of fly and vertebrate homologs of UNC-5, and the molecular pathways downstream of netrin reception. Given the conservation of the biology, it will be surprising if the regulatory events connected with netrins and their receptors are not also retained from nematodes to chordates.

Selected Reading

Chan, S.S.-Y., Zheng, H., Su, M.-W., Wilk, R., Killeen, M.T., Hedgecock, E.M., and Culotti, J.G. (1996). Cell *87*, 187–195.

Cho, K.R., and Fearon, E.R. (1995). Curr. Opin. Genet. Dev. *5*, 72–78.

Colamarino, S.A., and Tessier-Lavigne, M. (1995). Annu. Rev. Neurosci. *18*, 497–529.

Cooper, H.M., Armes, P., Britto, J., Gad, J., and Wilks, A.F. (1995). Oncogene *11*, 2243–2254.

Culotti, J.G. (1994). Curr. Opin. Genet. Dev. *4*, 587–595.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Mamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W., and Vogelstein, B. (1990). Science *247*, 49–56.

Harris, R., Sabatelli, L.M., and Seeger, M.A. (1996). Neuron *17*, 217–228.

Hedgecock, E.M., Culotti, J., and Hall, D.H. (1990). Neuron *2*, 61–85.

Ishii, N., Wadsworth, W.G., Stern, B.D., Culotti, J.G., and Hedgecock, E.M. (1992). Neuron *9*, 873–881.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S.-Y., Culotti, J.G., and Tessier-Lavigne, M. (1996). Cell *87*, 175–185.

Kennedy, T.E., Serafini, T., de la Torre, J.R., and Tessier-Lavigne, M. (1994). Cell *78*, 425–435.

Kolodziej, P.A., Timpe, L.C., Mitchell, K.J., Fried, S.A., Goodman, C.S., Jan, L.Y., and Jan, Y.N. (1996). Cell *87*, 197–204.

Lumsden, A.G.S., and Davies, A.M. (1983). Nature *306*, 786–788.

Mitchell, K.J., Doyle, J.L., Serafini, T., Kennedy, T.E., Tessier-Lavigne, M., Goodman, C.S., and Dickson, B.J. (1996). Neuron *17*, 203–215.

Ramon y Cajal, S. (1909) Histologie du Système Nerveux de l'Homme et des Vertébrés (Paris: A. Maloine), p. 661.

Schall, T. J., and Bacon, K. B. (1994). Curr. Opin. Immunol. *6*, 865–873.

Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M., and Tessier-Lavigne, M. (1994). Cell *78*, 409–424.

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C., and Tessier-Lavigne, M. (1996). Cell *87*, in press.

Skarnes, W.C., Moss, J.E., Hurtley, S.M., and Beddington, R.S.P. (1995). Proc. Natl. Acad. Sci. USA *92*, 6592–6596.

Shirasaki, R., Tamada, A., Katsumata, R., and Murakami, F. (1995). Neuron *14*, 961–972.

Shirasaki, R., Mirzayan, C., Tessier-Lavigne, M., and Murakami, F. (1997). Neuron *18*, in press.

Tessier-Lavigne, M., Placzek, M., Lumsden, A. G. S., Dodd, J., and Jessell, T. M. (1988). Nature *336*, 775–778.

Tucker, A., Varela-Echavarria, A. W., Puschel, A. W., and Guthrie, S. (1997). Neuron *18*, in press.

Vielmetter, J., Kayyem, J. F., Roman, J. M., and Dreyer, W. J., (1994). J. Cell Biol. *127*, 2009- 2020.

Wadsworth, W. G., Bhatt, H., and Hedgecock, E. M. (1996). Neuron *16*, 35–46.